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Assemblage of filamentous fungi associated with aculeate hymenopteran brood in reed galls

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ABSTRACT

Monotypic stands of common reed and the reed-gall-associated insect assemblages are distributed worldwide. However, fungi associated with these assemblages have not been characterized in detail. Here we examined 5200 individuals (12 species) of immature aculeate hymenopterans or their parasitoids collected at 34 sampling sites in Central Europe. We noticed fungal outgrowth on exoskeletons of 83 (1.60%) larvae and pupae. The most common host was eudominant *Pemphredon fabricii*. However, the less abundant aculeate hymenopteran reed gall inquilines were infected at higher prevalence, these included *Trypoxylon deceptorium*, *Trypoxylon minus*, *Hoplitis leucomelana* and *Hylaeus moricei* (all considered new host records). We identified three fungal species, *Penicillium buchwaldii* (72% of cases), *Aspergillus pseudoglaucus* (22%) and *Penicillium quebecense* (6%). When multibrooded nests were affected, only a part of individuals was infected in 62% of cases. The sampling site-specific infection rate reached up to 13%, thus fungal infections should be considered an important variable driving the abundance of gall inquilines. Infections of generalist host species were more frequent than those of reed gall specialists, suggesting that suboptimal conditions decreased the immunocompetence of non-specialized species, which only occasionally nest in reed galls and feed in reed beds.

1. Introduction

Entomopathogenic fungi are typically found in various lineages of chytrids, zygomycetes and ascomycetes (Samson et al., 1988). Numerous fungal infections affect insect species of economic importance, or may involve zoonotic species as in this study. Their life cycles are usually synchronized with insect host stages and environmental conditions (Shah and Pell, 2003). However, most of the research has been focused to several socially living species of insects or to the synanthropic species, and there is limited knowledge on the contribution of entomopathogens in the regulation of pest populations in agroecosystems and on the delivery of ecosystem services to agricultural production (Altieri, 1999; Gurr et al., 2003; Tscharntke et al., 2005; Meyling and Eilenberg, 2007). Thus, our improved understanding of the ecology of indigenous populations of entomopathogenic fungi is considered a prerequisite for the evaluation of their economic impact and for their consideration as non-market goods (Meyling and Eilenberg, 2007). Here we focus on species associated with cavity-nesting hymenoptera (bees and wasps) making their nests in galls of chloropid flies *Lipara* spp. on common reed *Phragmites australis* stems. Monotypic stands of common reed and the gall-associated assemblages are distributed worldwide. They swiftly colonize newly formed (post)industrial habitats and thus may serve as a ubiquitously available support for the establishment of entomopathogenic fungi. The reed-associated arthropod hosts may utilize reed as a food source (sap suckers, leaf-, pollen-, and phloem-feeding species) or as a nesting resource and shelter (stem borers, gall makers, and gall inquilines). Tewksbury et al. (2002) reported 160 species of reed-associated arthropods in Europe, but they found only 23 species of reed-associated arthropods in North America, where the subsp. *americanus* is native, but subsp. *australis* is considered an alien species.

Aspergilli, Mucorales and Penicillia are considered key fungi associated with honey bees (Gilliam and Prest, 1987; Gilliam et al., 1989; Kirpik et al., 2010). Stonebrood caused predominantly by *Aspergilus flavus* and chalkbrood caused by *Ascosphaera apis* are known as key pathogenic infections contributing to colony losses

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(Gilliam and Vandenberg, 1997). Interestingly, the resistance of honey bees to obligate parasitic fungi causing chalkbrood is dependent on host genotype, whereas the resistance to facultative parasitic fungi causing stonebrood is negligible (Evison et al., 2013). The epidemiology of stonebrood and chalkbrood is poorly understood, and high number of cases is probably undetected because the diseased brood is thought to be rapidly discarded by worker bees (Foley et al., 2014).

Despite the first record of fungi in aculeate hymenoptera was reported by Franciscan monk José Torrubia already in year 1749 (Samson et al., 1988), vast majority of hitherto performed studies of fungi infesting aculeate hymenoptera addressed nearly exclusively the honey bee *Apis mellifera*, other species of economic importance and ant mounds. Goerzen (1991) studied the microflora associated with healthy adult and larval alfalfa leafcutting bee *Megachile rotundata*, observing increased larval mortality when yeast and bacterial fermentation of provisions was followed by mould overgrowth. Solitary bees *Centris pallida* and *Anthophora* sp. were studied by Gilliam et al. (1984). Filamentous fungi colonizing the exoskeleton of dead *Mellipona subnitida* were reported by Ferraz et al. (2008). Enteric fungi of the paper wasp *Polistes hebraeus* were studied by Fouillaud and Morel (1995). Pathogenicity of selected fungal species to *Vespula vulgaris* and *Vespula germanica* was tested by Glare et al. (1996) and Harris et al. (2000). Strohm and Linsenmair (2001) found that the sphecid wasp *Philanthus triangulum* prolonges the resistance of its paralyzed malaxed prey (bees) to fungal infestation when compared to freeze-killed bees, but did not line their brood cells with any substances, which would prevent contamination by microbes similarly as it was reported from multiple colletid and halictid bees. Other mechanisms may apply, particularly metabolites of symbiotic bacteria of the crabronids and other invertebrate hosts may play an important protective role (Kaltenpoth et al., 2012). The cockroach parasitoid *Ampulex compressa* sanitizes host cuticule and the cocoon with a cocktail of nine antimicrobials, and uses also vaporous isocoumarine (*R*)-(-)-mellein to sanitize the nest by fumigation (Weiss et al., 2014). Also poor composition of the diet of bee larvae was experimentally shown to contribute to the pathogenicity of opportunistic fungi, such as *Aspergillus fumigatus* (Foley et al., 2012). Reliance on proper food supplementation is important to consider not only in relation to the food shortage, but also in relation to co-infections by parasites such as *Nosema ceranae*, which has been confirmed to impose an energetic stress (Mayack and Naug, 2009). Despite passive acquision of fungi from the soil and plant surfaces is usually suggested, Benoit et al. (2004) hypothesized that the mites, such as the honey bee parasitic mite *Varroa destructor* can harbor fungi and bacteria on their cuticle, including the pathogenic *Ascosphaera apis*, and also *Aspergillus* spp. and *Penicillium* spp., and thus may serve as their vectors. Aquino et al. (2013) in turn hypothesized, that aculeate hymenoptera serve as vectors of soil and airborne fungal species, suggesting that these insects should be eradicated in hospitals and other human-associated environments. Some fungal species associated with aculeate hymenopterans are considered symbiotic. Among them are *Amylostereum areolatum* and *Amylostereum chailletii*, which colonize mycangia of woodwasps *Sirex noctilio* and *Sirex nigricornis*. These fungal symbionts are injected during oviposition together with phytotoxic mucus into host pine trees. However, the presence of *Amylostereum* spp. is also considered a necessary pre-condition for a development of the nematode *Deladenus siricidicola*. This nematode serves as a biological control agent of *Sirex* spp., and displays a bicyclic life cycle including mycetophagous free-living and parasitic cycles (Olatinwo et al., 2013).

Insect galls represent stable microhabitats characterized by high humidity and limited air circulation, which is thus favorable for fungal proliferation and both accidental and obligate interactions of the gall insect with fungi (Bissett and Borkent, 1988). An example of such obligate mutualism is the colonization of openings of large *Lasioptera arundinis* galls by the fungus *Radulidium subulatum*. In this association the fungus allows the larva to penetrate into a stem, protects the larva against parasites and allows an easy exit of the imago, whereas the gall midge larvae and adults have structures allowing carrying the fungus, and thus facilitating spread of the fungus, and the larva induces reed gall formation (Yukawa and Rohfritsch, 2005). In non-obligate associations, the fungal assemblages found in galls are typically recruited from local endophytes present in adjacent tissues. Insect galls can affect the species composition of fungal endophytic species, and infecting fungi can negatively affect the fitness or even kill the insect present in the galls (Wilson, 1995; Lawson et al., 2014). It is notable that moulds of the genera *Penicillium* and *Aspergillus*, including the recently described taxa, are known as facultative gall associates, which can be explained by their ability to grow in habitats with limited availability of water. Seifert et al. (2004) reported *Penicillium cecidicola*, *Penicillium glabrum* and *Penicillium paxilli* in galls of Cynipidae species, *Penicillium dendriticum* in galls of unknown origin on *Eucalyptus* leafs, and *Penicillium erythromellis* and *P. pseudostromaticum* in galls induced by *Diplolepis rosae* on *Rosa sitchensis*.

In this study, we have characterized reed gall aculeate hymenopteran inquilines hosting fungal species. The superficial fungal growth was noticed when sampling immature aculeate hymenopterans for the purpose of taxonomic and ecological analyses. The diagnosis was based on the sequencing of multiple DNA loci of both, the primary isolates and the strains transferred to an artificial medium, and based on the phenotyping, which in combination allowed precise identification of the pathogens to the species level. Epidemiological data were provided.

2. Material and methods

2.1. Study area and sampling

The study specimens were collected at 34 sampling sites in the Czech Republic (33 sites) and Slovakia (1 site), Central Europe (48.62–50.71°N; 12.25–18.56°E). Six examined reed beds were located in nature reserves and near-natural habitats, other 28 examined reed beds were at (post)industrial sites. At each of the sampling sites, 200 reed galls were collected between 7 Feb and 23 Mar 2014, with the exception of two sites, where the total number of galls available onsite was less than 200 and thus only the available galls were collected. The chosen sampling period corresponded to the end of high air moisture period in the Czech Republic (September to February). It also reflected the life cycle of host organisms, as all of them survive the winter as larvae in a diapause. Larvae collected during the chosen sampling period can easily develop to adults under laboratory conditions, which is not as trivial for those collected prior the winter onset. Thus, sampling at the end of winter allowed comparing the viability of infected larvae with the uninfected ones.

Only galls older than 1 year (greyish or darker in appearance, usually without leaves and with the apex broken) were collected, because the study focused on aculeate hymenopterans, which preferentially (or, perhaps, exclusively) use the $\geqslant 1$ year old galls for their nesting. The collected galls were longitudinally cut, the healthy aculeate hymenoptera were allowed to rear and the specimens with macroscopic signs of fungal infections were collected for further analyses. All the aculeate hymenopteran specimens were identified to a species level. The detailed list of sampling sites